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14. ABSTRACT

The primary objective of the proposed research was to investigate the role of the Gadd45 family of genes (Growth Arrest & DNA Damage) in breast carcinogenesis. A research plan was designed taking advantage of an established breast cancer prone mouse models (i.e. MMTV-ras and MMTV-myc mice) that were crossed with Gadd45α deficient mice. Generation of these mice allowed for the investigation of the role Gadd45α plays in breast cancer formation/progression. Tumorigenesis was accelerated in Ras/Gadd45α-/- mice compared to Ras/Gadd45α+/+ mice. The median tumor onset for Ras/Gadd45α-/- mice was 6 months of age, compared to 8 months for Ras/Gadd45α +/+. In addition, histological examination and grading of the tumors show the Ras/Gadd45α-/- specimen with higher histological grades than the Ras/Gadd45α+/+. Upon irradiation treatment, there was a significant increase in the tumor incidence, where 100% of the Ras/Gadd45α-/- mice developed tumors by 8 months of age compared to 12 months of age for the Ras/Gadd45α+/+ irradiated mice. The tumor growth rate and histological grades of the Ras/Gadd45α-/- irradiated tumors was significantly higher than that of the Ras/Gadd45α+/+. In MMTV-Myc mice, we have demonstrated that the loss of Gadd45α contributes to the deceleration of tumor formation. The mechanisms for acceleration/deceleration of breast tumorigenesis are an area of future investigation.

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DNA Repair, Cell Cycle, Apoptosis

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Table of Contents

INTRODUCTION

The development of breast cancer is a multistage process. Alterations in multiple genes that control cell proliferation, survival and metastasis are known to cooperate in the development and progression of breast tumorigenesis. The overall objective of the proposed research was to investigate the role of the Gadd45 family of genes (Growth Arrest & DNA Damage) (Gadd45α and Gadd45β) in breast carcinogenesis, due to the fact that Gadd45 genes have been shown to play an important role in cell cycle control and response to anti-cancer agents. In order to achieve this, a research plan was designed taking advantage of established breast cancer prone mouse models (MMTV-v-Ras and MMTV-c-Myc) that have been crossed with Gadd45α deficient mice. Mating these mice with Gadd45 deficient mice, which are not prone to the development spontaneous mammary adenocarcinomas, allowed for the establishment of breast cancer prone mouse strains that are deficient for Gadd45α gene, which were used to investigate how the loss of Gadd45 promoted breast cancer formation or progression under different experimental settings (i.e. IR treatment). We hypothesized that the loss of Gadd45α would accelerate tumorigenesis. Expanding the focus of the research, our experimental plan included generation of tumor cell lines from primary tumor masses collected from the mice and characterization of the cellular and molecular pathways involved in breast carcinogenesis and determination of the role Gadd45 plays in these processes.

BODY

Task 1. Assess the effect of Gadd45α or Gadd45α/β deficiencies on oncogene driven breast carcinogenesis.

As reported in previous annual reports, the process of establishing mouse strains that express either oncogenic Myc (MMV-c-Myc) or activated Ras (MMTV-v-Ras) and are deficient for Gadd45α, along with appropriate control mice has been underway in our laboratory. Following proper PCR genotyping protocols, the mice were placed in the appropriate experimental treatment groups (i.e. No Treatment, IR Treatment) and monitored for tumor formation and progression. Once a tumor was detected, the tumor growth properties where monitored every other day for approximately 14 days, at which time the mouse was sacrificed according to standard protocols. Tumor measurements were taken with hand calipers to evaluate tumor volume (calculated Tumor Volume (mm3) = $(W^2 \times L)/2$, where W is width and L is length).

In MMTV-Ras mice, we have demonstrated that breast tumorigenesis was accelerated by the loss of Gadd45α (**Figure 1**) (statistical significance was calculated using the Mantel-Cox log rank test). A total of 26 mice were used for the Ras/Gadd45α-/- experimental group and 25 mice for both the Ras/Gadd45α+/- and Ras/Gadd45α+/+ groups. Gadd45α deficiency significantly accelerated median tumor onset, measured as the age at which 50% of females had developed tumors. The median tumor onset for Ras/Gadd45α-/- mice was 6 months of age, compared to 8 months for Ras/Gadd45 α +/+. Interestingly, the median tumor onset for the Ras/Gadd45α+/- mice was 7 months. There was also a significant increase in the tumor incidence in the Ras/Gadd45α-/- mice, with 92% of mice developing tumors

within twelve months, compared to 74% of control Ras/Gadd45α+/+ mice developing tumors during the same time period (**Table 1**) (statistical significance was calculated using the χ^2 test). The tumor incidence for the Ras/Gadd45α+/- was an intermediate 84%.

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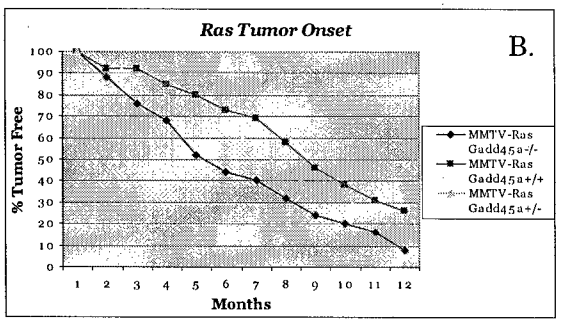

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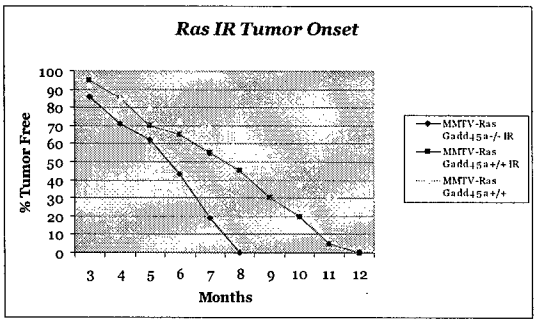

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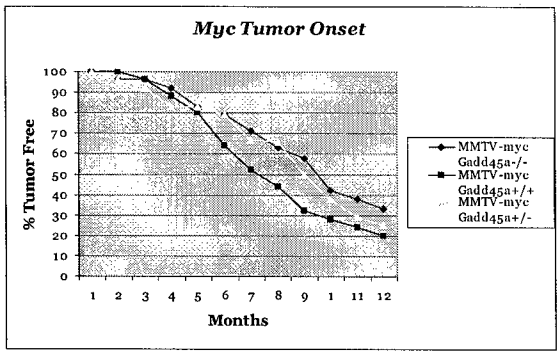


Figure 1: Tumor onset in MMTV-ras and MMTV-myc mice in the presence or absence of Gadd45α. Kaplan-Meier survival curve showing the proportion of tumor free female mice as a function of time for (A) Ras/Gadd45α, (B) Ras/Gadd45αIR and (c) Myc/Gadd45α.

Representative tumor samples from the two genotypes were fixed in 10% buffered formalin and embedded in paraffin, and sections were stained with Hematoxylin and Eosin for histological grading. Histological grading systems are used to describe the degree of malignancy of tumor specimens and also have been shown to have prognostic value for some tumor types, including breast cancer. Tumors were graded according to a scoring system of 0 to 3, where 0 represented normal cells. Grades 1 and 2 were assigned to tumors with irregular cellular shape, enlarged cellular size, increased nuclear size and increased nuclear/cytoplasmic ratio. Grade 3 tumors also exhibited multinucleated cells. Tumor specimens from Ras/Gadd45α-/- mice showed higher histological grades than that of the Ras/Gadd45 α +/+ (**Figure 2**).

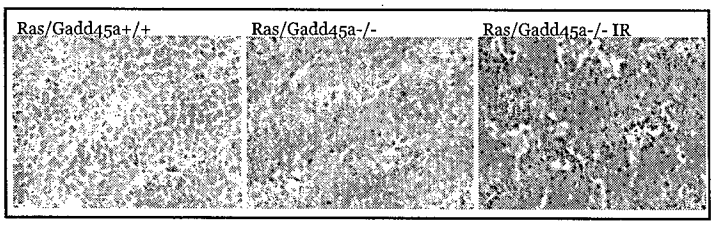


Figure 2: Histological evaluation of tumors arising from Ras/Gadd45α mice. Tissue was fixed in 10% buffered formalin and embedded in paraffin, followed by section staining with H&E. (40X magnification.)

Upon irradiation treatment of the Ras/Gadd45 α -/- mice, median tumor onset seemed unaffected, but there was a significant increase in the tumor incidence, where 100% of the mice developed tumors by 8 months of age compared to 12 months of age for the Ras/Gadd45 α +/+ irradiated mice (**Figure 1, Table 1**) (3 doses of 3 greys). The tumor histological grades of the Ras/Gadd45 α -/- irradiated tumors were higher than the Ras/Gadd45 α -/- (**Figure 2**).

In MMTV-Myc mice, intriguingly, we have observed that tumorigenesis was decelerated by the loss of Gadd45α (**Figure 1**) (statistical significance was calculated using the Mantel-Cox log rank test). A total of 24 mice were used for both the Myc/Gadd45α-/- and Myc/Gadd45α+/- experimental groups and 25 mice for the Myc/Gadd45α+/+ group. Gadd45α deficiency slowed median tumor onset to 8 months of age for Myc/Gadd45α-/- mice, compared to 7 months for Myc/Gadd45 α +/+. The median tumor onset for the Myc/Gadd45α+/- mice was unaffected at 7 months. There was a significant decrease in the tumor incidence in the Myc/Gadd45α-/- mice, with only 67% of mice developing tumors within twelve months, compared to 80% of control Myc/Gadd45α+/+ developing tumors during the same time period (**Table 1**) (statistical significance was calculated using the χ^2 test). The tumor incidence for the Myc/Gadd45α+/- was an intermediate 75%.

Although Gadd45α has a defined role in negative growth regulation, the mechanism for acceleration/deceleration of breast carcinogenesis in the Ras and Myc mice respectively remains unknown. Future experimental plans are designed to investigate these phenomenons.

Genotype	Median Tumor Onset	Tumor Incidence
Ras/Gadd45α-/-	6	92%
Ras/Gadd45α+/-	7	84%
Ras/Gadd45α+/+	8	74%
Ras/Gadd45α-/- IR	6	100%**
Ras/Gadd45α+/+ IR	8	100%
Myc/Gadd45α-/-	8	68%
Myc/Gadd45α+/-	7	75%
Myc/Gadd45α+/+	7	80%

*Table 1: Median Tumor Onset and Tumor Incidence of Ras/Gadd45α and Myc/Gadd45α mice. Median Tumor Onset was measured as the time at which 50% of the mice had developed tumor masses. Tumor Incidence was measured as the percentage of mice developing tumors within 12 months. **Indicates percentage of mice developing tumors within 8 months.*

Task 2. Characterization of breast tumors promoted by Gadd45 deficiency and oncogenic v-Ras or c-Myc.

Gadd45α has been shown to play a vital role in both cell cycle progression and apoptosis. It is also known that the growth of solid tumors depend on increased proliferation and decreased apoptosis. Given this, we chose to investigate the growth properties and apoptosis levels in the tumor specimens from the differing genotypes in order to characterize the observed tumorigenesis.

Animals were monitored biweekly for the formation tumors. Upon tumor formation, tumor growth was monitored for change in total volume over approximately 14 days or until the general health of the animal was compromised (as described above). Over this time, tumors of the Ras/Gadd45a-/- had an increased average growth rate of 110±10mg/day, compared to Ras/Gadd45α+/+ at 59±12mg/day (**Figure 3**). Interestingly, tumors from the Ras/Gadd45a+/- mice grew at an intermediate average growth rate of 79±13mg/day. Tumors arising from mice

following irradiation treatment had a marked growth rate increase to 124±15mg/day. Loss of Gadd45α decreased tumor growth rates in the Myc mice. The average tumor growth rate for the Myc/Gadd45α-/- was 54±9mg/day, Myc/Gadd45α+/- was 83±10 and Myc/Gadd45α+/+ was 91±11mg/day.

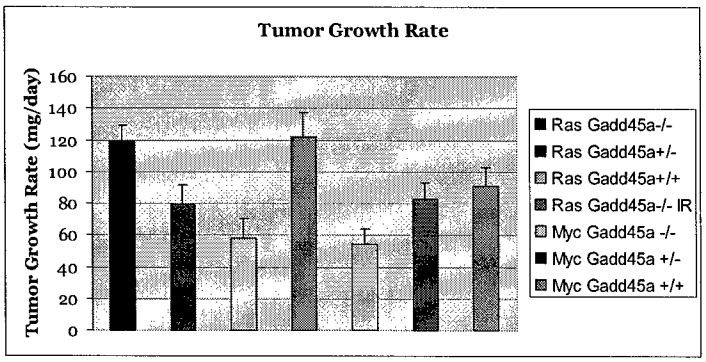
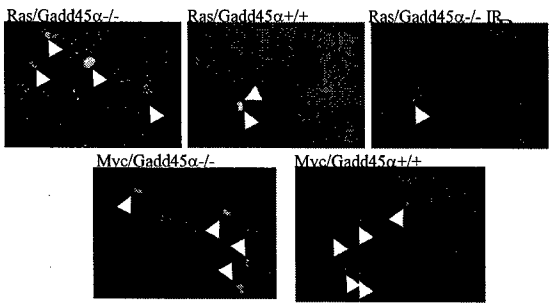


Figure 3: Tumor Growth Rates of tumors arising from Ras/Gadd45α and Myc/Gadd45α mice. Once a tumor was detected, tumor volume was calculated with caliper measurements as described in text.

To determine if the observed differences in the tumor growth rates were the result of differences in levels of apoptosis, TUNEL analysis was performed using tumor tissue sections from the varying genotypes. Ras/Gadd45α+/+ control tumors had very low levels of apoptosis (0.16% ±0.03%) (**Figure 4**). Ras/Gadd45α-/- had a significant increase in the level of apoptosis up to 0.36% ±0.08%, whereas the radiation treated tumor specimen had decreased levels at 0.02% ±0.02%. Tumor specimens from Ras/Gadd45α+/+ IR mice are currently being processed. Upon completion of these samples, further conclusions may be made.

It has been demonstrated in previous papers that MMTV-myc tumors have significantly higher levels of apoptosis than the MMTV-ras mice. Our results are consistent with these findings, where the Myc tumors display much greater levels of apoptosis then the Ras tumors. Myc/Gadd45α-/- and Myc/Gadd45α+/+ tumor samples displayed 2.4% ±0.8% and 3.1% ±0.9% respectively; whether these findings are significant remain to be determined via additional analysis (**Figure 4**).

A



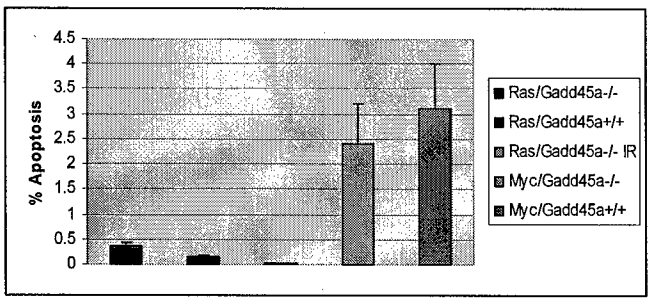


Figure 4: Analysis of Tumor Apoptosis. TUNEL assays were performed to determine the levels of apoptosis in varying genotypes of the Ras/Gadd45α and the Myc/Gadd45α mice. A. Representative pictures of TUNEL staining (40X magnification). B. The percentage of apoptosis was calculated by dividing the total number of positively labeled cells by the total number of PI stained cells in the field.

We are now in the process of investigating the cell cycle characteristics of tumor cells from the varying genotypes to determine if the changes of growth rates are the result of altered cell cycle kinetics. We hope to have those results within the next few months.

KEY RESEARCH ACCOMPLISHMENTS

* Generating MMTV-ras Gadd45α-/- mice.
* Generating MMTV-myc Gadd45α-/- mice.
* Generating proper control mice (i.e. MMTV-v-Ras or MMTV-c-Myc and Gadd45α+/+ or Gadd45α+/- and Gadd45α/β+/+ or Gadd45α/β+/-).
* Establishing experimental treatment groups.
* Development of tumor cell line.

REPORTABLE OUTCOMES

The major reportable outcome from the described research is the generation of the novel Ras/Gadd45α-/- and Myc/Gadd45α-/- mouse strains. The generation of these mice allow for future experimental plans that will continue to elucidate the molecular mechanisms of breast tumorigenesis.

In June 2005, the work from this grant was presented by Jennifer Tront, a graduate student in my laboratory. The presentation was in poster format at the 2005 Era of Hope Department of Defense Breast Cancer Meeting in Philadelphia, Pennsylvania. We have also submitted an abstract for consideration of a poster presentation at the AACR Advances in Breast Cancer Research Conference in La Jolla, California, along with a travel award application for Jennifer to attend this meeting.

These research findings largely contributed to the doctoral thesis of Jennifer Tront, who plans to graduate within the next calendar year with a Ph.D. in Molecular Biology & Genetics from the Temple University School of Medicine.

Lastly, these research findings are the basis for a newly formulated grant recently submitted to the NIH for consideration. The conclusions from this line of research were used as the preliminary data for continued investigation into the role of Gadd45 family of genes in breast tumorigenesis.

CONCLUSIONS:

Summary of Results: In MMTV-Ras mice, we have demonstrated that the loss of Gadd45α contributes to the acceleration of tumor formation. The median tumor onset for Ras/Gadd45α-/- mice was 6 months of age, compared to 8 months for Ras/Gadd45 α +/+. In addition, histological examination and grading of the tumors show the Ras/Gadd45α-/- specimen with higher histological grades than that of the Ras/Gadd45α+/+. The Ras/Gadd45α-/- tumors also displayed higher growth rates. Upon irradiation treatment of the Ras/Gadd45α-/- mice, median tumor onset seemed unaffected, but there was a significant increase in the tumor incidence, where 100% of the mice developed tumors by 8 months of age compared to 12 months of age for the Ras/Gadd45α+/+ irradiated mice. The tumor growth rate and histological grades of the Ras/Gadd45α-/- irradiated tumors was significantly higher than the Ras/Gadd45α+/+.

In MMTV-Myc mice, we have demonstrated that the loss of Gadd45α contributes to the deceleration of tumor formation. The median tumor onset for Myc/Gadd45α-/- mice was 8 months of age, compared to 7 months for Myc/Gadd45 α +/+. The Myc/Gadd45α-/- tumors also displayed lower growth rates.

Future experimental plans are designed to elucidate the mechanism by which loss of Gadd45α accelerates Ras tumor formation and decelerates Myc tumor formation.

Overall Significance of the Research: The overall objective of the proposed research was to investigate the role of the Gadd45 family of genes in breast carcinogenesis. Gadd45 proteins have been shown to play an important role in cell cycle control and response to anti-cancer agents. Results obtained from this research provide information that increase the understanding of the molecular basis of breast cancer development and may be utilized to design rational, novel therapies for treatment of breast cancer.

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